REMARKS

Reconsideration and allowance are respectfully requested.

Claims 1-4 and 13-14 are directed to the elected invention. Claims 5-12 and 15-20 are directed to nonelected inventions. Applicant urges reconsideration by the Examiner of the restriction requirement because the pending claims share the same special technical feature of germplasm comprising (i) a gene coding for the transglutaminase enzyme and (ii) one or more genes coding for wheat storage proteins, wherein said one or more wheat storage proteins comprise the preserved C-terminal motif LKVAKAQQL-AAQLPAMCR (SEQ ID NO: 11) and are selected from the group consisting of 1Bx7, 1By9, 1Dx5, 1Dy10, 1Ax2, 1Bx17, 1Ax1, 1Dy12, and HMW2. More specifically, upon allowance of an elected product claim, rejoinder of claims 10-11 and 15-20 is requested because they are directed to methods of making or using the product (i.e., flour).

The amendments are fully supported by the original disclosure and, thus, no new matter is added by their entry. Support for the flour having lower allergenicity than wheat flour may be found in the title ("low allergenicity"), the State of the Art section at pages 1-2 of the specification describing the allergenicity of wheat flour, and the elimination of amino acids responsible for allergenicity in wheat storage proteins (see SEQ ID NOS: 36 to 43). Amendments to method claim 11 are supported by page 1, lines 8-14, of the specification. Although "rising" ability in response to leavening agent (see the definition of "rise" from a dictionary attached as Exhibit A) and use as "food" are inherent in art-known definitions of flour used to make dough and baked products, the quoted terms are deleted because those limitations are not required for patentability.

It was alleged that search reports are not appropriate for printing on the face of an issued patent on page 5 of the Office Action. Applicant disagrees. No such limitation on the types of document that are listed on patents is found in the United States Code, Code of Federal Regulations, or Manual of Patent Examining Procedure. Search reports are printed documents and acknowledgment that they were considered by printing them on the patent's face is appropriate. Return of an initialed copy of the Form PTO-1449 without the lines through the search reports is requested. Otherwise, the Examiner is respectfully requested to provide legal authority in support of her allegation.

Further, the Information Disclosure Statement fully complies with the requirements and EP 1190624 should not have been lined through on the Form PTO-1449. M.P.E.P. § 609.05(b) states:

> Information which complies with requirements as discussed in this section but which is in a non-English language will be considered in view of the concise explanation submitted (see M.P.E.P. § 609.04(a)III) and insofar as it is understood on its face, e.g., drawings, chemical formulas, in the same manner that non-English language information in Office search files is considered by examiners in conducting searches. The examiner need not have the information translated unless it appears to be necessary to do so. The examiner will indicate that the non-English language information has been considered in the same manner as consideration is indicated for information submitted in English. The examiner should not require that a translation be filed by applicant. The examiner should not make any comment such as that the non-English language information has only been considered to the extent understood, since this fact is inherent.

Here, a concise explanation of the non-English foreign patents was provided in the Int'l Search Report citing them, the relevant portions of their disclosures that caused them to be cited, and the claims to which they apply. See M.P.E.P. § 609.04(a) III ("the requirement for a concise explanation of relevance can be satisfied by submitting an English-language version of the search report or action which indicates the degree of relevance found by the foreign office"). Therefore, EP 1190624 should have been considered prior to the first Office Action. To aid the Examiner, the U.S. patent documents listed on the attached Form PTO-1449 appear to be counterparts to the foreign patent documents.

Specification/Claim Objections

The title and abstract are amended to address the Examiner's objections.

Claim 1 recites optional modification of the one or more genes coding for wheat storage proteins by mutagenesis to eliminate allergenic amino acid sequences for food allergies to gluten. On page 3 of the Office Action mailed April 29, 2008, the Examiner admitted that "GROUPS I-X ARE LINKED BY CLAIM 1 AND CLAIM 4." In response to the restriction requirement, Applicant elected Group II (i.e., modification of the amino acid sequence SEQ ID NO: 36). But as admitted by the Examiner, claims 1 and 4 are generic; they link Group I (no modification) and Groups II-X (i.e., modifications of the

amino acid sequences SEQ ID NOS: 36-44). Therefore, it is proper that Applicant maintains them with their original scope to permit rejoinder of Group I-X in the future.

The plant may be a cereal or a leguminosa according to claim 1. Therefore, as regards claim 4, the plant can be rice, soybean or corn.

Applicant requests withdrawal of the objections.

35 U.S.C. 112 – Definiteness

Claims 1-4 were rejected under Section 112, second paragraph, as allegedly "indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention." Applicant traverses.

The phrase "low allergenic" is explained by reference to the specification. It refers to a lowering of allergenicity of the flour made from the seed/plant of the invention as compared to wheat flour. Table 5 of Applicant's specification demonstrates that flour of the invention has less immunoreactive gluten than wheat flour. Therefore, the claimed flour has lower allergenicity as compared to wheat flour.

The term "rising" is deleted from claim 1 because this limitation is not required for patentability. A person skilled in the art would understand that this refers to the ability of dough made from the claimed flour to trap therewithin gas produced in response to a leavening agent and to maintain an alveolar structure after baking. See page 1 of Applicant's specification. This ability of the dough is related to its viscoelasticity after water is added to the claimed flour. Even after amendment of the claims, this capability is inherent in the ability of the claimed flour to make dough and to result in a baked product.

Antecedent basis for "transglutaminase enzyme" is corrected. As noted by the Examiner, more than one transglutaminase enzyme is known in the art.

Finally, it is clear in claim 1 that "one or more" of the genes are optionally modified by mutagenesis to eliminate allergenic amino acid sequences for food allergies to gluten. One, more than one, all, or none of the allergenic amino acid sequences may be eliminated in accordance with the independent claim.

Applicant requests withdrawal of the Section 112, second paragraph, rejection because the pending claims are clear and definite.

35 U.S.C. 103 – Nonobviousness

A claimed invention is unpatentable if the differences between it and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art. *In re Kahn*, 78 USPQ2d 1329, 1334 (Fed. Cir. 2006) citing *Graham v. John Deere*, 148 USPQ 459 (1966). The *Graham* analysis needs to be made explicitly. *KSR v. Teleflex*, 82 USPQ2d 1385, 1396 (2007). It requires findings of fact and a rational basis for combining the prior art disclosures to produce the claimed invention. See id. ("Often, it will be necessary for a court to look to interrelated teachings of multiple patents . . . and the background knowledge possessed by a person having ordinary skill in the art, all in order to determine whether there was an apparent reason to combine the known elements in the fashion claimed by the patent at issue"). The use of hindsight reasoning is impermissible. See id. at 1397 ("A factfinder should be aware, of course, of the distortion caused by hindsight bias and must be cautious of arguments reliant upon ex post reasoning"). Thus, a prima facie case of obviousness requires "some rationale, articulation, or reasoned basis to explain why the conclusion of obviousness is correct." *Kahn* at 1335; see *KSR* at 1396. An inquiry is required as to "whether the improvement is more than the predictable use of prior art elements according to their established functions." Id. at 1396. But a claim that is directed to a combination of prior art elements "is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art." Id. Finally, a determination of prima facie obviousness requires a reasonable expectation of success. See *In re Rinehart*, 189 USPQ 143, 148 (C.C.P.A. 1976).

Claims 1-4 were rejected under Section 103(a) as allegedly unpatentable over Benmoussa et al. (WO 00/18927) in view of Arentz-Hansen et al. (J. Exp. Med. 191: 603-612, 2000), further in view of Schuhmann (U.S. Patent 6,517,874), and further in view of Whitelam (J. Sci. Food Agric. 68:1-9, 1995). Applicant traverses. In the election filed August 29, 2008, it was previously explained why the his claims were inventive:

> "It is urged that two essential features of the claimed invention (especially the elected flours) appear not to have been considered by the Examiner in her finding of lack of unity: the properties (i.e., low allergeni-

city and ability to rise) of the flours made from the seeds of the transgenic plants. An objective of the invention is to obtain flours that have low allergenicity (hence, not flours made from wheat) but are nevertheless capable of rising like wheat flours (see Figs. 23 and 24). Obtaining this objective was the result of the inventor's discovery that wheat storage protein having the C-terminal motif LKVAKAQQLAAQLPAMCR (SEQ ID NO: 11) is essential for non-wheat flours to rise when treated with transglutaminase. Thus, the invention uses a limited number of wheat storage proteins with the aforementioned motif that are capable of conferring the ability to rise on flours made from seeds of non-wheat plants such as rice and legumes.

Further, the Applicant has taken into account a further step to even improve the flours as aforementioned, namely targeted mutations of certain domains where amino acid residues are optionally mutated to minimize the allergenicity of the claimed flours. In order to make the flour immediately ready to use, the inventor has also expressed in the same seeds a transglutaminase. This direct expression of the transglutaminase in the seeds for making the flour led to the expression of an active enzyme in the seed.

The claimed invention thus permits the production of a non-wheat non-allergenic, rising flour that requires only the addition of yeast for rising (similar to wheat flour), but with the advantage that flours of the present invention **do not contain wheat allergens expressed in wheat seeds**.

The Examiner cites in her Office Action (page 5-6) the disclosures WO 98/08607 and US 6,517,874 as showing that the claims lack the same or corresponding special technical feature and thus do not relate to a single general inventive concept. It was stated, "Anderson O.D. (WO 98/08607) teaches a high molecular weight (HMW) glutelin from wheat that comprises SEQ ID NO: 11 . . . He teaches that HMW glutelin is important for flour quality, especially the viscoelasticity of the flour . . . He teaches transgenic plants expressing engineered HMW glutelin." But WO 98/08607 fails to teach or suggest the claimed invention for the following reasons:

1. WO 98/08607 allegedly teaches dough, preferably wheat dough with particular viscoelasticity properties and its use in products such as breads and noodles. See page 3, first paragraph, of the Summary of the Invention. Noodles are not relevant to the rising property of dough because the dough used for noodles is not reacted with yeast for rising. The examples are limited solely to wheat flour. No examples are provided of a non-wheat flour. In fact, the sole plant mentioned is wheat.
2. Moreover, WO 98/08607 teaches artificial HMW glutenin subunits. In particular, WO 98/08607 teaches that the modification of the number of certain repeats is related to viscoelasticity. See page 3, lines 8-20. Adding certain repeats in a number higher than the native protein improves the viscoelasticity properties of the wheat dough in which the modified protein

is expressed. The repeats that appear to be preferred (SEQ ID NOS: 1-5) are extremely rich in Glutamine.

WO 98/08607 does not even consider the problem of allergens in wheat flour. It discloses transforming wheat (which is allergenic per se) with an artificial HMW glutelin in which Glutamine rich repeats (linked to allergenicity) are added. This addition of Glu rich repeats is important for the invention disclosed in WO 98/08607 which makes flour with improved characteristics (i.e., viscoelastic properties) for the manufacturing of baked products and noodles (of course, rising is irrelevant to the latter). Therefore, WO 98/08607 teaches away from making modification in HMW glutelins that are not related to improving viscoelastic properties.

US 6,517,874 is also cited. The Examiner alleged that the patent "teaches that trans-glutaminase can be added to flour blends comprising as little as 1% wheat flour to produce a baking flour mixture with favorable properties." But US 6,517,874 fails to teach or suggest the claimed invention for the following reasons:

1. Although allegedly describing flour blends comprising **at least** 1% of wheat flour (wheat NEVER being completely absent) in column 2, US 6,517,874 does not exemplify a flour with less than 20% wheat flour (Example 2, Table 4) in the flour blends tested except in Example 1 where it is clearly shown that the absence of wheat flour in the blend makes the dough flat and hence not rising (see Table 1). In fact, the examples show that the rising properties of the flour blend treated with transglutaminase are already decreasing and not optimal with as much as 20% wheat flour in the blend.
2. Moreover, US 6,517,874 teaches that transglutaminase can be added at various stages of the blend or dough preparation. See column 3, lines 59-61. But it also clearly teaches by way of reiteration that the transglutaminase SHOULD NOT BE GROUND for best results.

Therefore, US 6,517,874 strongly teaches away from having transglutaminase present in the seeds before they are ground into flour or using a flour blend having less than 20% wheat flour even if the latter does not rise."

The cited documents would not result in the present invention because they fail to teach or make obvious that one of ordinary skill in the art would have known that the good viscoelasticity of dough made from flour of transgenic plants is related to cysteine residues at the terminal regions of HMW glutelins in the flour and this elasticity can be improved by repeating certain glutamine-rich domains. As explained above, viscoelasticity of dough (substantially a mixture of water and flour) is critical in its ability to "rise" when gas produced by leavening agent is trapped in alveolar structures. Otherwise, in the prior art, wheat flour is essential for obtaining rising flours. But Applicant's invention

further improves the viscoelastic characteristics of the dough from flour of transgenic plants by treatment with transglutaminase, which can be added after grinding the cereal or leguminosa for flour.

Arentz-Hansen also does gliadin proteins. Although mutant peptides are made, these mutations were not introduced into a storage protein nor was it demonstrated that the mutation would reduce the protein's allergenicity if expressed in transgenic plants. In fact, investigation of the gliadins disclosed by Arentz-Hansen shows that they do not even contain SEQ ID NO: 11 (see Arentz-Hansen et al., Gut, 46:46-51, 2000, which is attached as Exhibit B). In particular, Fig. 2 of Exhibit B proves that the preserved C-terminal motif LKVAKAQQLAAQLPAMCR is absent from the gliadin proteins disclosed by Arentz-Hansen. SEQ ID NO: 11 in a requirement of the storage proteins comprising the claimed flour.

The optional mutations that are made mandatory in certain dependent claims further lower allergenicity of the claimed flour. They act on sites that are potentially allergeneic on the storage proteins expressed in seeds of the transgenic plants. They would not be obvious in view of the cited documents.

The cited documents would not result in the claimed flour because Benmoussa increases the potential allergenicity of the flour by repeating glutamine-rich domains and not mutating the allergenic domains, Arentz-Hansen does not teach or make obvious a wheat storage protein comprising SEQ ID NO: 11, and Schuhmann does not eliminate wheat from its flour or preserve rising ability when the proportion of wheat is lowered. Among other failures, it is worth noting that none of the cited documents teaches or makes obvious preserving LKVAKAQQLAAQLPAMCR (SEQ ID NO: 11) in storage proteins encoded by transgenes derived from wheat because that C-terminal domain is linked to the effective action of transglutaminase. The latter would not be expressed from a transgene in the seed because the prior art teaches away from the presence of transglutaminase in seeds prior to grinding. Further, these failures establish that the prior art does not provide a reasonable expectation of success. Therefore, Applicant's claims are patentable.

Since the combination of cited documents does not render obvious Applicant's invention as represented by independent claim 1, claims 2-4 that depend from claim 1 are also not rendered obvious by the cited documents because all limitations of the independent claim are incorporated in its dependent claims. See M.P.E.P. § 2143.03 citing *In re Fine*, 5 USPQ2d 1596 (Fed. Cir. 1988).

Withdrawal of the Section 103 rejection is requested because the claims would not have been obvious to one of ordinarily skill in the art when this invention was made.

Conclusion

Having fully responded to the pending Office Action, Applicant submits that the claims are in condition for allowance and earnestly solicit an early Notice to that effect. The Examiner is invited to contact the undersigned if additional information is required

Respectfully submitted,

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; to remove and replace (tiles) on a roof **d** : to cut, break, ravel, take out, or undo (stitches) in sewing ; separate (as a garment) into its parts **2 a** : to slash or slit with or as if with a sharp blade (*ripped* up his waistcoat to feel if he was not wounded —Daniel Defoe) **b** *archaic* : to tear open (an old sore or grievance) **3** *archaic* : to recall to notice or reopen (as a closed issue or an unpleasant business) **4** : to utter violently (as an oath) ; burst out with ; spit out (*ripped* out vituperation, cursing, and blasphemy) ~ *vi* **1** : to pull or tear apart ; REND (the strain was too great; the sleeve *ripped* away from the coat) **2** : to move unchecked ; proceed without restraint ; rush headlong (a smart convertible coupe came *ripping* up the short steep drive —Christopher Morley) **3** : to burst out with violent or profane utterance — usu. used with *out* (*ripped* out with an oath) **syn** see TEAR — **rip into** : to tear into like a buzz saw ; ATTACK (*ripped into* his antagonist with fury) — **rip up the back** ; to assail with hostile comment esp. behind the victim's back ; BACK-BITE

²rip \"\ *n* **-s 1** ; a rent made by ripping ; a torn place ; a gap left by a seam giving way ; TEAR **2** ; a cut of wood along the grain (as by a ripsaw) **3** *dial Brit* : RUSH, SPEED

³rip \'rip\ *Scot var of* ²REAP

⁴rip \"\ *n* **-s** [perh. fr. ³*rip*] **1** ; a body of water made rough by the meeting of opposing tides or currents ; TIDE RIP **2** ; a current roughened by passing over an irregular bottom — used esp. of tidal currents and sometimes of currents in rivers; compare UNDERTOW

⁵rip \"\ *n* **-s** [perh. alter. of ²*rep*] **1** ; a worn-out worthless horse (left the spavin-legged old ~ standing there —Bruce Siberts) **2** ; a reckless or dissolute person ; LIBERTINE, RAKE (his elder brother was a bit of a ~ —Ngaio Marsh)

rip *abbr* **1** ripieno **2** ripped

RIP *abbr* **1** [L *requiescat in pace*] may he (she) rest in peace **2** [L *requiescant in pace*] may they rest in peace

ri·par·i·an \rə'perēən, (')rī[ī]p-, -pa(a)r-,-pār-\ *also* **ri·par·i·al** \-ēəl\ *adj* [L *riparius* riparian + E *-an* or *-al* — more at RIVER] **1** ; of or relating to or living or located on the bank of a watercourse (as a river or stream) or sometimes a lake (~ vegetation) (~ states) (~ scenery) **2** ; LITTORAL

²riparian \"\ *n* **-s** ; one that lives or has property on the bank of a river

riparian right *n* ; the right of one owning riparian land to have access to and use of the shore and water — compare LITTORAL RIGHT, WATER RIGHT

ri·par·i·ous \-ēəs\ *adj* [L *riparius*] ; RIPARIAN

rip cord *n* **1** ; a cord by which the gasbag of a balloon may be ripped open for a limited distance to release the gas quickly and so cause immediate descent **2** ; a cord or wire pulled manually or automatically in making a descent to release the pilot parachute which lifts the main parachute out of its container ready to open **3 a** ; a cord inserted longitudinally in a sheathed electric cable for use in ripping the sheath at the ends for easier removal (as in making connections) **b** ; an electric cord consisting of two individually insulated wires readily separable by ripping usu. for a short distance to make a connection

rip current *n* [*ripp*] ; a strong surface current flowing outward from a shore — called also *riptide*

¹ripe \'rīp\ *adj* **-ER/-EST** [ME, fr. OE *ripe*; akin to OS *rīpi* ripe, MD *ripe*, OHG *rīfi*; derivative fr. the root of E *reap*] **1** ; fully grown and developed ; MATURE: **a** ; ready for reaping or harvesting (~ grain) (a ~ field) **b** *of fruit* (1) ; having mature seed (2) ; fully developed and so usable as food **c** ; mature enough for use as cuttings — used of stems or other plant parts **d** *of timber or a forest* ; ready to be cut **e** ; free from budding cells — used of a yeast **2** ; having the full development and powers of maturity: **a** ; having full mental and physical maturity **b** ; having mature knowledge, understanding, or judgment ; CONSUMMATE, PERFECTED (a ~ scholar) (~ wisdom) **c** ; stemming from thorough consideration or reflection ; based on full deliberation (they deal with many subjects and are characterized by ~ reflection and consummate mastery of style —*Encyc. Americana*) **3** ; marked by maturity or fullness of time: **a** ; exhibiting full mental or physical powers (a ~ time of life) (a ~ age) **b** ; of advanced years ; LATE (lived to the ~ age of 90) **c** ; fully arrived ; SUITABLE (the time seemed ~ to proceed to evangelization —Kemp Malone) **4** ; ready for some action or purpose ; fully prepared for some use or object ; fit for consummation (a state of affairs ~ for axis exploitation —H.M.Sachar) (the classical type of monopoly capitalism ~ for public ownership —*New Statesman & Nation*) (here is a mixed-up character, ~ for the analyst —Lucy Crockett) **5** ; brought by aging to full flavor or to the height of desirability as food or drink ; MELLOW (~ cheese) (a ~ port) (~ venison) **6** ; ready to discharge ; MATURED — used of an abscess or boil **7** *archaic* ; DRUNK — used in the phrase *reeling ripe* **8** ; ruddy, plump, or full like ripened fruit (the invitation of ~ young lips) **9** ; due or ready for action, trial, or payment (as a lawsuit or a claim) **10** ; sufficiently developed to be removed by surgery — used of a cataract in the eye **11 a** ; ready to be discharged — used of eggs **b** ; containing ripe eggs or spermatozoa — used of a fish; compare GREEN

²ripe \"\ *vb* **-ED/-ING/-S** [ME *ripen*, fr. OE *riplan*, fr. *ripe* ripe] *chiefly dial* ; RIPEN, MATURE

³ripe \"\ *vb* **-ED/-ING/-S** [ME *ripen*, fr. OE *rīpan*; akin to OHG *roufen* to pluck, ON *ruppa*, *rupla*, Goth *raupjan* to pluck, ON *rǫgr* tuft of hair — more at RUG] *vt* **1** *chiefly Scot* **a** ; to make a thorough search of **b** ; to subject to thorough examination or investigation **2** *chiefly Scot* ; to steal from ; RUB **3** *chiefly Scot* ; to clear of something that obstructs ; clean out **4** *chiefly Scot* ; to break up or remove stones from (rough ground) ~ *vi*, *chiefly Scot* ; to make a search

⁴ripe \"\ *chiefly dial var of* reap

⁵ripe \"\ *n* **-s** [ME, fr. L *ripa* — more at RIVER] *archaic* ; RIVERBANK, SEASHORE

ripe·ly *adv* [ME, fr. *ripe* + *-ly*] **1** *obs* ; with mature deliberation **2** ; in a ripe manner ; with mature or developed appearance ; AMPLY

rip·en \'rīpən, -p²m\ *vb* **ripened** \-pənd,-p²md\ **ripening** \-p(ə)niŋ\ **ripens** \-pənz,-p²mz\ [*ripe* + *-en*] *vi* **1** ; to grow ripe ; become mature (as of grain, fruit, or a microorganism) **2** ; to approach or come to full development ; arrive at completeness or perfection ; become fit for use, for action, or for an appropriate purpose **3** ; to become ready to discharge — used of an abscess or boil **4** *of a photographic emulsion* ; to increase in average grain size as a result of physical treatment (as prolonged heating) or in sensitivity as a result of chemical treatment ~ *vt* **1** ; to make ripe (the grower may . . . ~ the fruit in transit or on arrival by means of ethylene —G.L.Jenkins & W.H.Hartung) (time had ~*ed* his life and mellowed its fruits —Van Wyck Brooks) **2** ; to bring to maturity, completeness, or perfection ; cause full development of ; fit or prepare for some use or purpose: as **a** ; to sour (cream) by bacterial action as a prelude to churning for butter to reduce fat loss and improve the flavor of the finished product **b** ; to age or cure (cheese) to develop characteristic flavor, odor, body, texture, and color **c** ; to improve flavor and tenderness of (beef or game) through the action of enzymes in the meat during a period of refrigeration **3** ; to cause to become ready to discharge — used of an abscess or boil **syn** see MATURE

rip·en·er \'rīp(ə)nə(r)\ *n* **-s** ; one that ripens

ripe·ness \'rīpnəs\ *n* **-ES** [ME *ripenes*, fr. OE *rīpnes*, fr. *rīpe* *ripe* + *-nes* -ness — more at RIPE] ; the quality or state of being ripe ; MATURITY, COMPLETENESS, PERFECTION

riper *comparative of* RIPE

ripe rot *n* ; decay of ripe fruit caused by a fungus; *esp* ; UTTER ROT

ripest *superlative of* RIPE

ripgut \'ə,ə\ *or* **ripgut grass** *n* [*rip* + *gut*] ; a troublesome weedy European grass (*Bromus rigidus*) adventive in California

ri·pi·o·lous \rə'pikələs, (')rī[ī]p-\ *adj* [fr. L *ripa* bank + E *-i-* + *-colous*] ; RIPARIAN

ri·pid·o·lite \rə'pidᵊl,īt, rī'p-\ *n* **-s** [G *ripidolith*, irreg. fr. Gk *rhipid-*, *rhipis* fan + *-o-* + *-lith*] ; a variety of chlorite

ri·pie·nist \rə'yānəst\ *n* **-s** [*ripieno* + *-ist*] ; one that plays a ripieno

¹ri·pie·no \-ē(,)nō\ *adj* [It, filled up, stuffed, supplementary, fr. *ri-* re- (fr. L *re-*) + *pieno* full, fr. L *plenus* — more at FULL] **1** ; of or relating to a musical instrument or performer serving solely to swell the mass of an orchestra ; SUPPLEMENTARY **2** ; TUTTI

²ripieno \"\ *n*, *pl* **ripie·ni** \-ē(,)nē\ *or* **ripienos** [It, fr. *ripieno*, adj.] ; a supplementary musical instrument or performer or the group playing the ripieno part — compare CONCERTINO

¹ri·poste *also* **re·post** \rə'pōst, rē'p-\ *n* **-s** [F *riposte*, alter. of *risposte*, fr. It *risposta*, lit., answer — more at RISPOSTA] **1** ; a fencer's quick return thrust following a successful parry **2** ; a retaliatory verbal sally ; REPORT (the critic's ~ might be that most of these stories are machine-made too —Harrison Smith) **3** ; a retaliatory maneuver or measure ; COUNTERATTACK (the raid upriver was a successful ~ to the enemy's earlier attack)

²riposte *also* **repost** \"\ *vb* **-ED/-ING/-S** [F *riposter*, fr. *riposte*] *vi* **1** ; to make a riposte in fencing **2** ; to deliver a verbal counterthrust ; make a telling retort (the two men argued and *riposted* —Janet Flanner) **3** ; to deliver a counterblow or counterattack (the general evaded pursuit and *riposted*) ~ *vt* ; to deliver (a verbal counterthrust) in reply ; RETORT

rip panel *n* ; a strip in the upper part of the fabric of a balloon or semirigid or nonrigid airship which is torn off when immediate deflation is desired

ripped *past of* RIP

¹rip·per \'rip(ər)\ *n* **-s** [*rip* + *-er*] **1** ; a worker who rips: as **a** ; one who opens seams to prepare them for resewing **b** ; an operator of a ripsaw or variety saw **c** ; a member of a fish-dressing gang who opens the belly of fish and removes the viscera **2** ; a tool, device, or machine that rips: as **a** ; a long bar or thin steel blade used to remove damaged roofing slates and notched for drawing nails **b** ; a device for opening stitched seams in cloth **c** ; RIPSAW **d** ; a machine with revolvable knives for cutting millboard **e** ; a road machine to break up worn or disintegrated pavement **f** ; a subsoiler or chisel implement that loosens surface soil and subsoil **3** ; any of the larger blocks on which the first few drawings are effected in wiredrawing **4 a** ; an excellent example or instance of a kind ; HUMDINGER (a ~ — American musical comedy in the highest gear —*Newsweek*) **b** ; a wild or reckless person **5** *chiefly NewEng* ; ²BOOSTER **6** ; a madman or criminal who slashes his victims with a knife

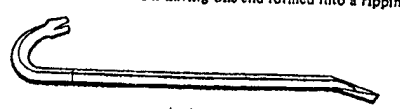
²ripper \"\ *adj* ; designed to make drastic changes in a governmental agency for purely partisan purposes (~ legislation) (~ bill) (~ amendment)

rip·pot *or* **rip·pit** \'ripət\ *n* **-s** [origin unknown] *dial* ; a noisy quarrel ; FUSS, UPROAR

rip·pi·er *or* **rip·i·er** \'ripē(r)\ *n* **-s** [*ripp* + *-ier*] *archaic* ; a fish peddler

rip·ping \'ripiŋ, -pēŋ\ *adj* [prob. fr. pres. part. of ²*rip*] ; GRAND, SWELL (also some — letters from you — O.W.Holmes †1935)

ripping bar *n* ; a steel bar having one end formed into a ripping



ripping bar

chisel and the other end shaped like a gooseneck with a claw for pulling nails

ripping chisel *n* ; a long slender chisel sometimes with a slightly bent and cleft cutting end used for cleaning mortises or for heavy prying

ripping cord *n* ; RIP CORD 1

ripping iron *n* ; RAVELHOOK

rip·ping·ly *adv* ; SPLENDIDLY

ripping panel *or* **ripping strip** *n* ; RIP PANEL

ripping punch *n* ; a tool with a rectangular point used in a punching machine for crosscutting metal plates

ripping saw *n* ; RIPSAW

ripping size *n* ; the size of rough wood stock required for the production of a finished product

¹rip·ple \'ripəl\ *vt* **-ED/-ING/-S** [ME *riplen*; akin to MD *repelen* & *repen* to ripple, MLG *répelen*, MHG *reffen* to ripple, OHG *rīfilōn* to saw] **1** ; to remove (seeds) from flax or hemp with a ripple **2** ; to draw (flax) through a ripple

²ripple \"\ *n* **-S** [ME *repylle*, *ryppyll*; akin to MLG *répel* ripple, OHG *rīfila* saw, *rīfilōn* to saw — more at ²RIPPLE] ; a large instrument like a comb for removing seeds and other matter from flax or hemp

³ripple \"\ *vi* **-ED/-ING/-S** [ME *replen*, of Scand origin; akin to Norw *ripla* & *ripa* to scratch, Sw *repa* — more at REAP] *dial Brit* ; to scratch slightly

⁴ripple \"\ *n* **-s** *dial Brit* ; a slight cut ; SCRATCH

⁵ripple \"\ *vb* **-pled; -pled; rippled; rippling** \-p(ə)liŋ\ **ripples** [perh. fr. ²*rip* + *-le*] *vi* **1 a** ; to become fretted or lightly ruffled on the surface (as water) ; become covered with or form in small waves or undulations (a blue river *rippled* into the bay — Israel Zangwill) (the ripened cornfields *rippled* up to the doorsteps of the cottages —Flora Thompson) (his lean, sun-bronzed upper body *rippled* all over with long, graceful muscle —Frank Yerby) **b** ; to flow in small waves (the brook *rippled* onward below her) **c** ; to fall in soft undulating folds or wavy lines (the cloth *rippled* to the floor) **2** ; to flow with a light rise and fall of sound or inflection (laughter *rippled* over the audience) **3** ; to move with an undulating motion or so as to cause ripples (the canoe *rippled* through the water) **4** ; to run irregularly through a crowd, group, or population (had watched discontent ~ through the seaports and back country —Oscar Handlin) ~ *vt* **1** ; to stir up small waves on (water) ; move or disturb lightly (a moderate breeze was *rippling* the lagoon —Ernest Beaglehole) **2** ; to impart a wavy motion or appearance to (began to stretch and ~ his muscles —*Time*) **3** ; to utter or play with a slight rise and fall of sound ; make a light rapid cadence or melody of (*rippling* a boogiewoogie beat on the piano —Noel Houston)

⁶ripple \"\ *n* **-s 1 a** ; a shallow stretch of running water in a stream roughened or broken by rocky or uneven bottom **b** (1) ; the fretting or ruffling of the surface of water (as by wind) (2) ; a small wave **c** ; a small wave propagated by *wave* **2** ; something resembling or suggesting a ripple of water: as **a** ; RIPPLE MARK 2 **b** ; a soft fold (as in a full skirt) or a wavy outline (as in a hat brim) **c** ; CHATTER MARK 1 **d** ; a sound like that of rippling water (a ~ of laughter) (a ~ of conversation) **3** ; RIPPLER 1 **4** ; a slight fluctuation in the intensity of an otherwise steady electrical current

ripple fire *n* ; the discharge of rockets in quick succession

ripple grass *n* ; a ribgrass (*Plantago lanceolata*)

ripple index *n* ; the horizontal distance from crest to crest divided by the vertical distance from crest to trough of the ripples on a ripple-marked surface

rip·ple·less \'ripəl(ə)s\ *adj* ; having no ripples ; GLASSY, SMOOTH

ripple mark *n* **1 a** ; the undulating surface of a ridge or trough produced in incoherent granular material (as loose sand) by wind, currents of water, or the agitation of waves **b** ; one of the ridges on such a surface **2** ; a striation across the grain of wood esp. on the tangential surface caused by stories of cambial and other cells — **ripple-marked** \'ə,ə\ *adj*

rip·pler \'rip(ə)lə(r)\ *n* **-S** ; one that ripples; *esp* ; RIPPLE

rip·plet \'rip(ə)lət\ *n* **-S** ; a small ripple

ripple voltage *n* ; the alternating component of unidirectional voltage from a rectifier or generator

ripple weld *n* ; a weld having a rippled surface

ripple-weld \'ə,ə\ *vt* (*ripple weld*) ; to unite by means of a ripple weld

rip·pling·ly *adv* ; in a rippling manner ; WAVILY

rip·pon \'ripən\ *n* **-S** [fr. *Rippon* (Ripon), Yorkshire, England] *archaic* ; a horseman's spur

¹rip·rap \'ri,prap\ *n* [fr. obs. *riprap* sound of rapping, redupl. of ²*rap*] **1** ; a foundation or sustaining wall of stones thrown together without order (as in deep water, on a soft bottom, or on an embankment slope to prevent erosion) **2** ; stone used for riprap

²riprap \"\ *vt* ; to form a riprap in or upon ; strengthen or support with a riprap (*riprapped* the breakwater with stone)

riprapping *n* **-S** ; a riprap foundation or wall

rip-roaring \'ri,prōriŋ, -prōr-\ *adj* [alter. of *rip-roarious*] ; noisily excited or exciting ; HILARIOUS

rip-roarious \(')ri,prōrēəs, -prōr-\ *adj* [²*rip* + *-roarious* (as in *uproarious*)] ; HILARIOUS

rips *pl of* RIP, *pres 3d sing of* RIP

ripsack \'i,ə\ *n* [²*rip* + *sack*] ; GRAY WHALE

¹ripsaw \'i,ə\ *n* [²*rip* + *saw*] ; a coarse-toothed saw for cutting wood in the direction of the grain — compare CROSSCUT SAW

²ripsaw \"\ *vi* ; to saw (wood) in the direction of the grain

rip·sawyer \'rip+,\ *n* [²*ripsaw* + *-yer* (as in *sawyer*)] ; a worker who uses a ripsaw

ripsnorter \'i,ə\ *n* [²*rip* + *snorter*] **1** ; a violently energetic or noisily outspoken person ; someone using slambang methods (that venerable old ~ defended the rights of the clergy to drive fast horses — Phil Stong) **2** ; something extreme ; HUMDINGER (yesterday's performance was a ~ for gaiety, vigor, and general run-around —Virgil Thomson) — compare SNORTER — **ripsnorting** \'i,ə\ *adj*

riptide \'i,ə\ *n* [*rip* + *tide*] **1** ; RIP CURRENT **2** ; a situation in which confused overwhelming forces play on someone : a destructive vortex (creators and culture heroes who crack up in a ~ of alcohol —Richard Chase)

rip track *n* ; a siding on which railroad cars are given minor repairs

rip·u·ar·i·an \ripyə'werēən, -wa(ə)r-,-wār-\ *adj*, *usu cap* [L *Ripuarius* Ripuarian + E *-an*] ; of or relating to a group of Franks settling in the 4th century on both banks of the Rhine near the present city of Cologne

²ripuarian \"\ *n* **-s** *cap* ; a Ripuarian Frank

rip van win·kle \,rip,van'wiŋkəl\ *n* **-s** *usu cap R&V&W* [after *Rip Van Winkle*, fictional ne'er-do-well in the Catskill mountains of N.Y. state in colonial days who slept for 20 years, as portrayed in a story of *The Sketch Book* (1820) by Washington Irving †1859 Am. writer] ; someone not alert to current conditions (some *Rip Van Winkle* in an obscure corner . . . who has read nothing for four years —*Manchester Guardian Weekly*)

rir·o·ri·ro \,rirə'rē(,)rō\ *n* **-S** [Maori] ; GRAY WARBLER

ri·sa·la *or* **res·sa·la** \rə'sälə\ *n* **-s** [Hindi *risāla* troop, fr. Per, fr. *A risālah* mission] ; a troop of irregular cavalry in the Anglo-Indian army

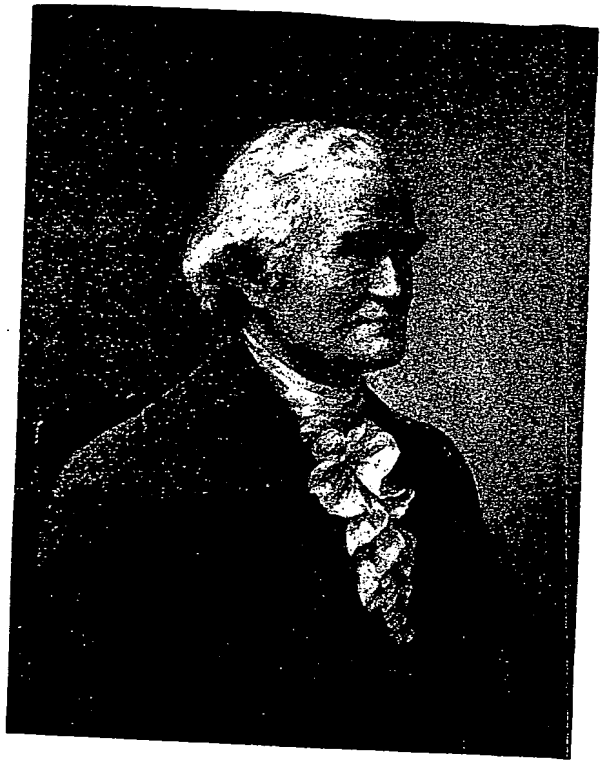
ri·sal·dar *or* **ris·sal·dar** \rə'səl,dür, 'risəl,-\ *or* **res·sal·dar** \rə'səl,dür, 'resəl,-\ *n* **-S** [Hindi *risāldār*, fr. Per *risāladār*, fr. *risāla* + *-dār* holder — more at BHUMIDAR] ; a native commander of a risala in the Anglo-Indian army

risco *n* **-S** [It *risco*, *rischio*, *rischio*] *obs* ; RISK, VENTURE

¹rise \'rīz\ *vb* **rose** \'rōz\ *or archaic* **rise** *or dial* **riz** \'riz\ **ris·en** \'riz²n\ *or dial* **riz**; **ris·ing** \'rīziŋ\ **ris·es** \'rīzəz\ [ME *risen*, fr. OE *rīsan*; akin to OHG *rīsan* to rise, climb, fall, ON *rīsa* to rise, Goth *urreisan* to get up, L *orīri* to rise, *rivus* brook, stream, Gk *ornynai* to urge on, cause to rise, *oros* mountain, Skt *arnas* wave, *rṣva* high, *raya* stream, Hitt *ardi* he up from lying, kneeling, or sitting **b** ; to get up from sleep or from one's bed **c** ; to get back on one's feet after a fall **d** ; to regain standing after a lapse, disgrace, or failure **e** (1) ; to stand erect (as of a terrified person's hair) (2) ; to resume an upright position (as of flattened grass or grain) **2** ; to come back to life ; return from death or the grave (witnesses who ate and drank with him after he *rose* from the dead — Acts 10:41 (RSV)) **3 a** ; to go to war ; take up arms ; launch an attack ; make insurrection (the people of Boston *rose* and seized all of the Dominion officers who could be found —Viola F. Barnes) — usu. used with *against* (the Lord will cause your enemies who ~ against you to be defeated before you —Deut. 28:7 (RSV)) **b** *obs* (1) ; to break camp (2) ; to withdraw a besieging force **4 a** *obs* ; to show respect ; DEFER — used with *up* **b** ; to respond warmly ; show enthusiasm ; APPLAUD, CHEER — usu. used with *to* (the audience *rose* to his verve and wit) **5** ; to end or a session ; ADJOURN (when the committee *rose* on Friday the clerk had read through section 203 —*Congressional Record*) **6 a** ; to move up from the horizon ; climb the skies ; come up (a pale sun *rose* in lowering skies) — opposed to *set* **b** ; to come in view (as of a ship at sea) above the horizon or to appear larger on nearer approach **7 a** ; to ascend into the air ; move upward (smoke *rose* quietly from cottage chimneys all through the valley below) **b** ; to grow taller ; increase in height (~ to heights unusual for other trees) **8** ; to swell in size or volume ; reach a higher level (the river *rose* rapidly with the heavy rains) (becomes an island each time the tide *rises*) (a blister *rose* at the burn) (broad dough *rises*) **9** ; to extend upward ; grow in process of construction ; incline or reach above other objects (at a little distance above him *rose* a small butte —Oliver La Farge) (octagonal towers ~ a story higher than the main body of the structure —*Amer. Guide Series: Texas*) (a meeting place or assembly hall will ~ —Sidonie M. Gruenberg) (between the valleys of the gorge ~ miniature mountain peaks —*Amer. Guide Series: La.*) **10 a** ; to become lifted up or raised ; swell with joy ; increase in cheer, hope, or courage ; become elated (spirits *rose* as the danger passed) **b** ; to increase in fervor ; grow heated or ardent ; INTENSIFY (members of his staff watched indignation ~ in him —Stewart Cockburn) **11** ; to move aloft ; become lifted higher ; go up ; SOAR (the curtain *rose* on a lovely set) (birds *rose* all around in alarm) **12 a** ; to come up to the surface (as of the water or the ground) (a diver *rose* near him in the water) (the spring ~*s* cool and fresh from great depths) **b** ; to move up through the water to take food or bait (trout were *rising* hungrily) **13** *of locked-up printing type* **a** ; LIFT **b** ; to work up **14 a** ; to attain a higher level ; gain in vigor, clarity, grace, or effectiveness (his painting *rose* to a fresh expressiveness and revealed a subtler, gentler insight) (*rose* to heights of passionate eloquence) **b** ; to increase in quantity or number (funds available for investment *rose* sharply —R.P.Edmunds) (cotton acreage *rose* over 50 percent —*Americana Annual*) **c** ; to advance in rank, position, or esteem (*rose* to the rank of brigadier general of cavalry when still in his twenties —J.H.Easterby) **d** ; to increase in price ; grow dearer (the cost of paper *rose*) **e** ; to become higher in pitch or louder (her voice *rose* then in a shrill crescendo) **15 a** ; to grow stronger or more resolute (his courage *rose* as difficulties multiplied about him) **b** ; to increase in force or rate of speed (the wind *rose* rapidly) (storms *rose* often to wild fury) **16** ; to take place ; HAPPEN, OCCUR (then *rose* a little circumstance that was to have far-reaching results) **17 a** ; to attain existence ; come on the scene ; become born ; APPEAR (search and you will see that no prophet is to ~ from Galilee —Jn 7:52 (RSV)) (great regimes *rose*, based upon the irrational and negative in man's nature —M.W.Straight) **b** *archaic* ; to spring up ; GROW — used of a plant **18** ; to follow as a consequence ; become derived ; ORIGINATE, RESULT (wars had *risen* out of incidents more trivial —L.C.Douglas) **19** ; to gain currency ; CIRCULATE (a rumor *rose* in city hall circles that the mayor would resign) **20 a** ; to have source or origin ; SPRING (the river ~*s* in the foothills) **b** ; to have a beginning (great nations ~ and fall) **21** ; to exert oneself to meet a challenge or provocation ; show oneself equal to a demand or test ; prove adequate — usu. used with *to* (their ministerial leaders *rose* ably to the occasion with consummate theological arguments —*Amer. Guide Series: Conn.*) **22** ; to become raised (as of a vowel) ~ *vt* **1** ; to cause to rise; *esp* ; to lure (a fish) to rise **2** *chiefly dial* ; to make higher ; INCREASE — used of price **3** *archaic* ; to reach the top of ; SURMOUNT

syn RISE, ARISE, ASCEND, MOUNT, SOAR, TOWER, ROCKET, LEVITATE, SURGE can mean, in common, to move or come up from a lower to a higher level. RISE is the most general, inter-

Exhibit A



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Gut 2000;46:46–51

Production of a panel of recombinant gliadins for the characterisation of T cell reactivity in coeliac disease

E H Arentz-Hansen, S N McAdam, Ø Molberg, C Kristiansen, L M Sollid

Abstract

***Background/Aims*—Coeliac disease is a chronic intestinal disorder most probably caused by an abnormal immune reaction to wheat gliadin. The identification of the HLA-DQ2 and HLA-DQ8 as the molecules responsible for the HLA association in coeliac disease strongly implicates a role for CD4 T cells in disease pathogenesis. Indeed, CD4 T cells specific for gliadin have been isolated from the small intestine of patients with coeliac disease. However, identification of T cell epitopes within gliadin has been hampered by the heterogeneous nature of the gliadin antigen. To aid the characterisation of gliadin T cell epitopes, multiple recombinant gliadins have been produced from a commercial Nordic wheat cultivar.**

***Methods*—The α-gliadin and γ-gliadin genes were amplified by polymerase chain reaction from cDNA and genomic DNA, cloned into a pET expression vector, and sequenced. Genes encoding mature gliadins were expressed in *Escherichia coli* and tested for recognition by T cells.**

***Results*—In total, 16 α-gliadin genes with complete open reading frames were sequenced. These genes encoded 11 distinct gliadin proteins, only one of which was found in the Swiss-Prot database. Expression of these gliadin genes produced a panel of recombinant α-gliadin proteins of purity suitable for use as an antigen for T cell stimulation.**

***Conclusion*—This study provides an insight into the complexity of the gliadin antigen present in a wheat strain and has defined a panel of pure gliadin antigens that should prove invaluable for the future mapping of epitopes recognised by intestinal T cells in coeliac disease.**

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Keywords: gliadin; coeliac disease; small intestine; protein expression; T lymphocyte; gluten

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Coeliac disease is an inflammatory disorder of the small intestine characterised by atrophy of the villi and hyperplasia of crypt cells.[1 2] The disease is precipitated in genetically susceptible people on ingestion of wheat gluten and the related proteins of rye and barley. Several lines of evidence indicate that the development of the disease is due to an abnormal immune response to gluten. The identification of HLA class II molecules DQ2 and DQ8 responsible for the HLA association observed in coeliac disease[3–8] implicates a role for coeliac disease T cells in disease pathogenesis. This is further supported by the demonstration that T cells extracted from intestinal biopsy specimens of patients with coeliac disease, but not of control patients, contain a population that are coeliac disease positive and αβ T cell receptor positive and specific for gliadin. These gut derived T cells are conspicuous in that the vast majority recognise gliadin peptides when presented by DQ2 or DQ8.[9–11] Gluten comprises numerous related proteins which together provide a nitrogen store for the germinating seed. On the basis of their solubility in aqueous alcohol, gluten can be separated into the gliadins (soluble) and the glutenins (insoluble). The gliadins are monomer molecules which can be further divided into three classes on the basis of sequence (α-, γ-, and ω-gliadins).[12] Gliadins in each class differ slightly in sequence,[13] and in a single wheat variety at least 45 different gliadins have been identified at the protein level.[14] The glutenins consist of large polymeric structures that are formed as the result of intermolecular disulphide bonds. Most T cells isolated from biopsy specimens challenged with gluten appear to recognise the alcohol soluble gliadin fraction.[9] Interestingly, this recognition of gliadin by intestinal T cells is dependent on deamidation, a modification known to be promoted in the acidic environment during pepsin digestion. However, deamidation is most probably mediated in vivo in a specific fashion by the enzyme tissue transglutaminase (tTG).[15] This enzyme mediated deamidation introduces negative charges into gliadin and increases the binding affinity of gliadin peptides for DQ2. A better understanding of the processes that lead to the modification and T cell recognition of gliadin peptides, and which ultimately cause disease, requires that the intestinal T cell response to the gliadin antigen be characterised at a molecular level. Identification of T cell epitopes in gliadin has been complicated by the enormous microheterogeneity of the complex gliadin antigen and the difficulty in purifying antigens with a defined sequence. To aid the characterisation of T cell epitopes, we have cloned, sequenced, and expressed a panel of gliadins in *Escherichia coli* and tested them for their ability to stimulate

Abbreviations used in this paper: PCR, polymerase chain reaction; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis; tTG, tissue transglutaminase.

Exhibit B

gut derived T cell clones of patients with coeliac disease.

Materials and methods

GLIADIN GENE

The plasmid containing the γ-gliadin gene pW1621 was a gift from A Rafalski (DuPont Agricultural Biotechnology, Wilmington, Delaware, USA).

ISOLATION OF DNA AND MRNA AND SYNTHESIS OF CDNA

The Nordic autumn wheat strain Mjoelner was the source of genomic DNA and mRNA. The wheat endosperm was harvested 30 days after flowering. Genomic DNA was phenol extracted from the endosperm or blades. mRNA was isolated from the endosperm using the Dynabeads mRNA DIRECT kit (Dynal, Oslo, Norway). For cDNA synthesis, RNA was eluted from the beads and added to a mixture containing 1 mM dNTP (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK), 1 μM oligo$(dT)_{15}$ primer (Promega, Madison, Wisconsin, USA), 20 mM dithiothreitol (Gibco-BRL, Paisley, Scotland, UK), 20 U RNasin (Promega), and 200 U Moloney murine leukaemia virus (Gibco-BRL) and incubated for one hour at 37°C. The enzymes were inactivated by heating at 65°C for five minutes.

AMPLIFICATION AND CLONING OF GLIADIN GENES

Oligonucleotide primers (Eurogentec, Herstal, Belgium) were designed for amplification of gliadin genes coding for mature proteins without leader sequences. The α-gliadin specific primers had the sequences: sense primer 5' GCT ATG GAT CCA TAT GGT TAG AGT TCC AGT GCC and antisense primer 5' GCA TCA AGC TTC ATC GAT AGT TAG TAC CGA AGA TGC C. The γ-gliadin specific primers had the sequences: sense primer 5' GCT ATG GAT CCA TAT GAA TAT CCA GGT CGA CCC and antisense primer 5' GCA TCA AGC TTC ATC GAT ATT GGC CAC CAA TGC CCG C. In addition to start and stop codons, *Hin*dIII and *Nde*I restriction sites were included in the primer sequences for cloning and expression purposes.

For polymerase chain reaction (PCR) amplification, about 100 ng genomic DNA or cDNA was used in a reaction mixture containing 1 mM each dNTP (Amersham Pharmacia Biotech), 200 nM each primer, 2 mM $MgCl_2$, 1.25 U *Taq* DNA polymerase (Promega), and 0.008 plaque forming units polymerase (Stratagene, La Jolla, California, USA). The denaturation, annealing, and polymerisation temperatures for the first five cycles were 94°C, 45°C, and 72°C respectively. In the following 30 cycles the annealing temperature was increased to 55°C. Purified PCR fragments were cloned into the *Nde*I and *Hin*dIII site of the pET17xb vector according to the instructions in the manufacturer's manual (Novagen, Madison, Wisconsin, USA). The ligation mixure was used to transform competent Nova Blue cells (Novagen), and recombinant plasmids were isolated using Wizard Plus SV Minipreps (Promega).

DNA SEQUENCING

Cycle sequencing of gliadin clones was performed on PCR products amplified with T7 vector specific primers using the Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham Pharmacia Biotech) according to the manufacturer's manual. Two vector specific sequencing primers were used for both α-gliadin and γ-gliadin: T7 promoter primer, 5' TAA TAC GAC TCA CTA TAG GG; T7 terminator primer, 5' GCT AGT TAT TGC TCA GCG G. The internal α-gliadin specific primers were: (*a*) 5' GCA TGG ATG TTG TAT T, (*b*) 5' CTG CAA TAC AAC ATC C, (*c*) 5' GCA GGG ATG TTG TCT T, (*d*) 5' TTG CAA GAC AAC ATC C, (*e*) 5' TGC TGA CAA CAC AAT T. The internal γ-gliadin specific primers were: (*a*) 5' ATT CTT GCA TGG GTT CA; (*b*) 5' TGA ACC CAT GCA AGA AT. Sequencing products were run on an ABI Prism 377XL DNA sequencer (Perkin Elmer, Norwalk, Connecticut, USA).

PRODUCTION OF RECOMBINANT GLIADINS IN *E COLI*

Plasmids containing gliadin inserts of appropriate lengths were transformed into competent BL21(DE3)pLysS cells (Novagen) containing a single copy of the T7 polymerase gene under the control of the inducible lac UV5 promoter. Freshly plated single colonies were grown in 1 litre cultures of Luria-Bertani medium containing 100 μg/ml carbenicillin and 34 μg/ml chloramphenicol at 37°C. At a culture density of A_{600} = 0.6, isopropyl β-D-thiogalactoside (Sigma, St Louis, Missouri, USA; 0.4 mM) was added and the cultures were incubated for a further 18 hours. At harvesting, cell pellets obtained by centrifugation (15 minutes at 650 *g* and 30 minutes at 2520 *g*) were resuspended in preheated (60°C) 70% ethanol and incubated at 60°C for two hours. Bacterial cell debris was then removed by centrifugation (30 minutes at 14 500 *g*), and two volumes of 1.5 M NaCl was added to the supernatant to precipitate the gliadin proteins. The solution was incubated at 4°C overnight to allow complete precipitation of the gliadins. The precipitate was collected by centrifugation (30 minutes at 14 500 *g*), rinsed briefly with distilled water, and dissolved in 8 M urea/0.4 M NH_4HCO_3. The proteins were analysed using one dimensional sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) with 12% gels and staining with Brilliant Blue R250 (Sigma). Protein was determined with a BCA protein assay (Pierce, Rockford, Illinois, USA) using commercial gliadin (Sigma) as a standard.

PREPARATION OF RECOMBINANT GLIADINS FOR T CELL ASSAYS

The solubilised gliadins were dialysed against 0.1 M NH_4HCO_3 overnight to remove the urea. Gliadin proteins are hardly soluble in physiological salt solutions, but can be made soluble through limited enzymatic proteolysis and thereby become suitable as antigens in T cell assays. The recombinant gliadins used in T

cell assays were therefore digested with either α-chymotrypsin (C-4129; Sigma; 1:100, w/w) at 37°C in 0.1 M NH_4HCO_3 until dissolved or pepsin (P7012; Sigma; 1:100, w/w) at 37°C in 0.1 M acetic acid, pH 1.8, for three hours. The chymotrypsin digested recombinant gliadins (125 μg/ml) were incubated with 250 μg/ml guinea pig tTG (T-5398; Sigma) at 37°C for two hours in phosphate buffered saline with 0.8 mM $CaCl_2$ whereas the pepsin treated recombinant gliadins were heat treated in the digestion buffer at 98°C for two hours before addition to T cell assays.

T CELL ASSAY

The following gut derived DQ2 restricted T cell clones of patients with coeliac disease were used: CD 380 E-27, CD 387 E-34, CD 412 R-3,[10] CD 370 R-2.3, and CD 412 R-5.32 (Ø Molberg, unpublished). The last two clones were cloned using a protocol identical with that for the published clones. In brief, gliadin specific T cell lines were established from small intestinal biopsy specimens challenged in vitro with a peptic-tryptic digest of gliadin. The gliadin specific T cell lines were cloned at limiting dilution to establish gliadin specific T cell clones.

T cells (5×10^4) were added to 5×10^4 antigen presenting cells (HLA matched allogenic Epstein Barr virus transformed B lymphoblastoid cell lines irradiated with 80 Gy) that had been incubated for 16–20 hours with either untreated or tTG treated recombinant gliadins (50 μg/ml) in a volume of 100 μl RPMI 1640 (Life Technologies, Rockville, Maryland, USA) supplemented with 15% pooled and heat inactivated human serum. The assay was performed in 96-well U bottom plates (Costar, Cambridge, Massachusetts, USA), and T cell proliferation was measured as [^{3}H]thymidine incorporation 48–72 hours after the antigenic stimulation. Activation of the 60.6 T cell transfectant[15] was measured as interleukin-2 release after an 18 hour incubation of 2.5×10^4 transfectant cells with 5×10^4 non-irradiated antigen pulsed DQ2 positive B lymphoblastoid cells. Murine interleukin-2 was quantified by time resolved fluorometry using Delfia reagents (Wallac, Turku, Finland) and the rat anti-murine interleukin-2 antibodies JES6-1A12 and JES6-5H4 (PharMingen, San Diego, California, USA).

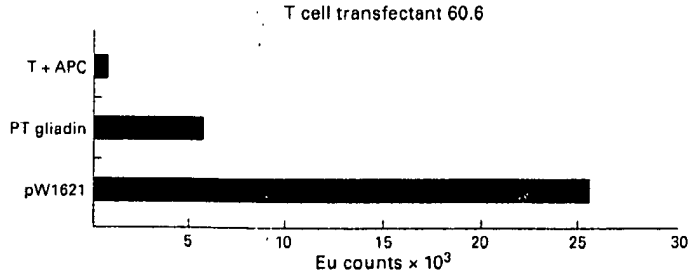

Figure 1 Recognition of pepsin treated pW1621 recombinant gliadin by the T cell transfectant 60.6. Pepsin-trypsin treated crude gliadin from Sigma (PT gliadin) was used as positive control. T cell activation was measured as interleukin-2 release and is given as arbitrary europium (Eu) counts. T+APC, T cells plus antigen presenting cells.

Results

ESTABLISHMENT OF THE EXPRESSION SYSTEM

As yet only one gliadin epitope that is recognised by gut derived DQ2 restricted T cells of patients with coeliac disease has been described.[17] This γ-gliadin epitope is created after deamidation of a critical glutamine residue by either heat treatment in an acidic environment[17] or enzymatically by tTG.[15] To validate the suitability of the selected *E coli* expression system for the characterisation of T cell epitopes, we cloned a γ-gliadin gene (pW1621) that contains the published epitope into the pET17xb expression vector and expressed it in BL21(DE3)pLysS cells. Sequencing of plasmids obtained from PCR amplification of the pW1621 plasmid with subsequent cloning of the PCR product into the pET vector identified several positions that diverged from the published sequence (data not shown). In two out of seven pW1621 clones, single substitutions were found that are likely to represent misincorporation of nucleotides by the DNA polymerase. This allows a rough estimate of the PCR error rate in this system to be calculated as 1 in 2775. In contrast with the PCR introduced errors, seven substitutions were found in all clones and are probably due to sequencing errors reported in the sequence published in 1987. Notably, these substitutions cause a change in five amino acids from the deduced protein sequence—that is, residues 192 S→C, 255 I→M, 256 D→H, 264 H→Q, 265 E→Q; numbering relates to protein with leader sequence. Attempts to use bacterial lysates as T cell antigen failed because the T cell clones reacted non-specifically with bacterial material (data not shown). It was therefore necessary to purify the gliadin proteins from the bacterial lysates. For the purification, we adapted a protocol of A Tatham (personal communication) in which the gliadins are extracted with aqueous alcohol and precipitated with NaCl. From lysates of bacteria expressing the pW1621 gene, we obtained a relatively pure protein of apparent mass 36 kDa as assessed by SDS/PAGE (data not shown). This recombinant γ-gliadin protein was then digested with pepsin and acid/heat treated and tested for its ability to stimulate a T cell transfectant specific for this epitope.[15] Reassuringly, the recombinant protein efficiently stimulated this transfectant (fig 1).

AMPLIFICATION, CLONING, AND SEQUENCING OF GLIADIN GENES

We next wanted to express a panel of different gliadin genes coding for proteins to which the Norwegian population is exposed. Thus cDNA and genomic DNA were isolated from a wheat strain (Mjoelner) commonly grown in Norway. The α-gliadin genes were amplified from both cDNA and genomic DNA whereas the γ-gliadin genes were amplified from genomic DNA only. Altogether, 16 independent α-gliadin clones were sequenced. Eleven unique sequences were obtained. Notably, only one of the deduced protein sequences (α-2) identically matched a previous entry in the Swiss-Prot data bank (accession number

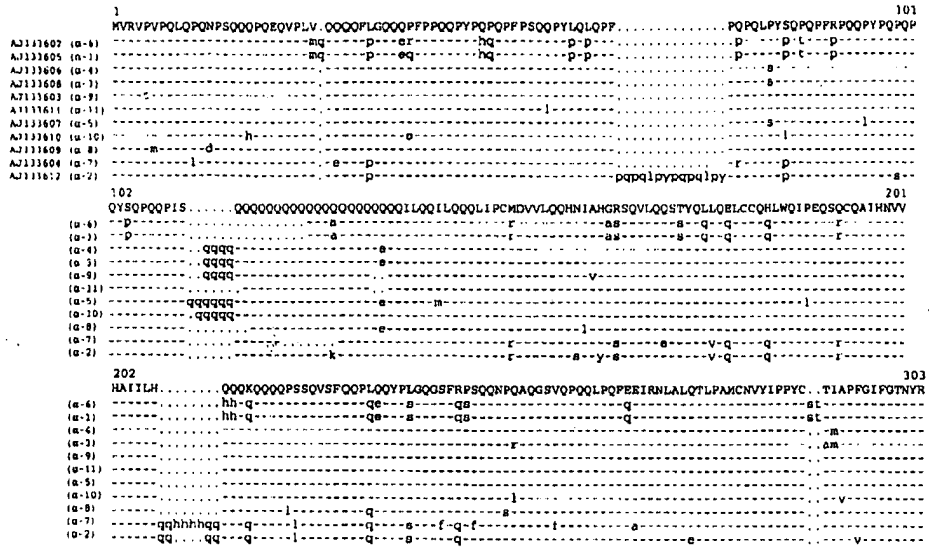

Figure 2 Amino acid sequence alignment of the α-gliadin clones. The EMBL accession numbers of the DNA sequence and the clone names are indicated. A consensus amino acid sequence is given above the alignment. The N-terminal M and the C-terminal Y and R are non-gliadin sequences that are introduced as part of the expression vector. The sequences of the six N-terminal residues and the eight C-terminal residues are determined by the primers used for the PCR amplification.

P18573[18]) (fig 2). Sequencing of the first 400 bases on the 5' end of 15 independent clones obtained from a PCR with γ-gliadin specific primers gave seven sequences unrelated to gliadin, and eight gliadin sequences. Complete sequencing of two of these clones identified one mature γ-gliadin not previously described in the database (GenBank accession number AJ133613) and one pseudogene.

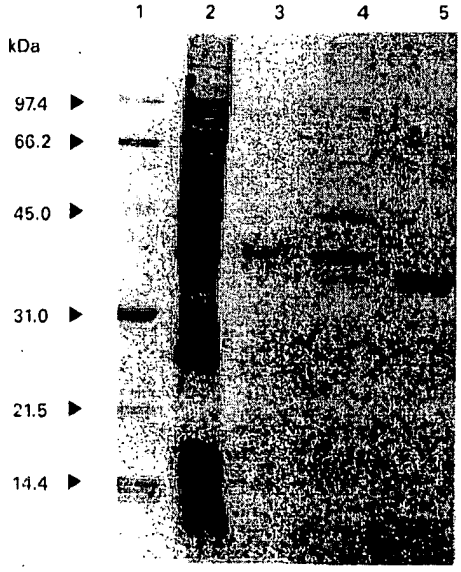

Figure 3 SDS/PAGE of the recombinant gliadins, before and after purification. Lane 1, molecular mass markers; lane 2, bacterial lysate from batch 1; lane 3, purified recombinant gliadins from batch 1; lane 4, lysate from bacteria expressing a single gliadin (α-11, not present in batch 1); lane 5, purified α-11. The bacterial lysates were harvested 18 hours after induction with isopropyl β-D-thiogalactoside.

EXPRESSION AND T CELL RECOGNITION OF BATCHES OF RECOMBINANT α-GLIADINS

Two batches, each containing nine randomly picked α-gliadin clones, were produced in 1 litre cultures. SDS/PAGE analysis of the purified gliadins disclosed bands with apparent masses of 36–40 kDa (fig 3). After chymotrypsin digestion, the complex gliadins either untreated or treated with tTG, were tested for recognition by four different gliadin specific gut derived T cell clones in proliferation assays. Both batches contained material that was recognised by all the T cell clones, but only after treatment with tTG (fig 4).

EXPRESSION AND T CELL RECOGNITION OF SINGLE RECOMBINANT α-GLIADINS

The nine gliadins from batch 1 were then expressed in individual 1 litre cultures. Both the apparent molecular mass (data not shown) and the yield for each of the gliadins (table 1) varied between the different clones. DNA sequencing of the seven clones that produced protein showed five unique sequences, α-1, α-2, α-3, α-4, and α-5 (fig 2 and table 1). These five gliadins were then tested for their ability to stimulate four different T cell clones, three of which were previously shown to stimulate batch 1. Four of the five recombinant gliadins failed to stimulate any of the T cell clones. However, one recombinant gliadin (α-2) stimulated all of the T cell clones. Stimulation of the T cell clones by this recombinant was entirely dependent on treatment with tTG (fig 5).

Discussion

We have cloned a panel of gliadin genes with distinct sequences from a wheat strain used for food production and expressed their respective

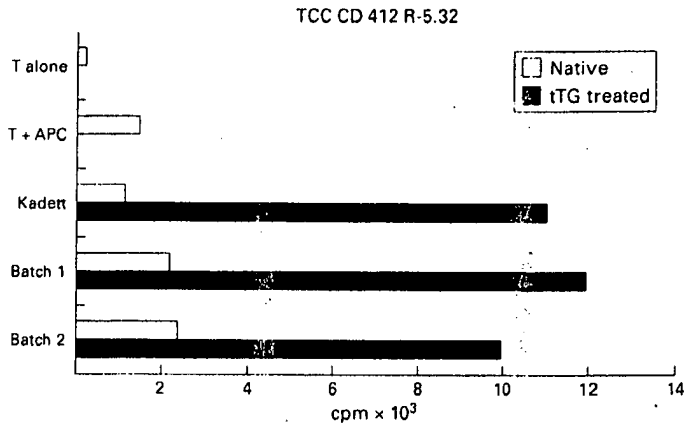

Figure 4 Recognition of batches of chymotrypsin digested and tissue transglutaminase (tTG) treated recombinant α-gliadins by the gut derived DQ2 restricted T cell clone (TCC) CD 412 R-5.32. Batches 1 and 2 each contain nine α-gliadin clones. Crude gliadin prepared from the wheat strain Kadett was used as a positive control. Similar data were obtained from the other T cell clones tested—that is, CD 380 E-27, CD 387 E-34, and CD 370 R2.3. T+APC, T cells plus antigen presenting cells.

Table 1 Protein-expressing clones from batch 1

α-gliadin clone	*Protein yield (mg/l culture)*	*Deduced molecular mass (kDa)*
α-1*	28.3	30.6
α-2*	19.6	33.7
α-3	21.0	32.2
α-4	24.7	32.2
α-5	10.7	32.5

*Clones present twice in this batch.

proteins in *E coli*. The gliadins were easy to produce and purify. Furthermore, a recombinant gliadin was shown to efficiently stimulate antigen specific T cells in in vitro assays. The availability of recombinant gliadins with defined sequences should facilitate the identification of T cell epitopes that are involved in the development of coeliac disease.

T cells are likely to be involved in the pathogenesis of coeliac disease. This notion is supported by the strong disease association with DQ2 and DQ8 and the presence of gliadin specific and DQ2 or DQ8 restricted T cells in the small intestinal mucosa of patients with coeliac disease. T cells recognise antigens in the form of peptide fragments (10–15 amino acids) bound to HLA molecules. Small variations in peptide antigen sequence may affect T cell recognition.[19] Notably, gliadin is not a simple protein but is, in fact, a complex mixture of distinct proteins that differ by minor variations in amino acid sequence. The sequence variation found between these proteins is likely to be important for their recognition by T cells.

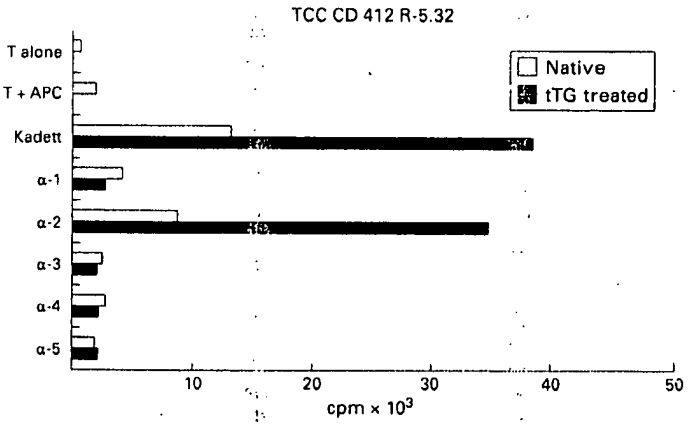

Figure 5 Recognition of one of the recombinant gliadin subtypes (α-2) after chymotrypsin digestion and tissue transglutaminase (tTG) treatment of the T cell clone (TCC) CD 412 R-5.32. Similar data were obtained from the other T cell clones tested—that is, CD 380 E-27, CD 412 R-3, and CD 370 R2.3. T+APC, T cells plus antigen presenting cells.

The isolation of a single species of a gliadin protein from wheat flour by biochemical methods is difficult and laborious,[20 21] and there is always a danger that the isolated protein actually comprises several different species with indistinguishable physicochemical properties. Furthermore, gliadins have stretches of repetitive glutamines and high contents of proline which pose particular problems for N- and C-terminal amino acid sequencing. Moreover, gliadin peptides are difficult to sequence by tandem mass spectrometry because they often fragment poorly. The challenge to identify the sequence of epitopes is further compounded by the fact that most gliadin T cell epitopes recognised by gut T cells contain deamidated glutamines.[17] These reasons motivated us to express recombinant proteins. The knowledge of the exact sequence of the whole protein provides a scaffold, which enables efficient sequence identification from limited mass spectrometry data. Thus the use of recombinants should allow the information of the sequence of stimulatory and non-stimulatory gliadins to be combined with the full power of mass spectrometry for sequence analysis to identify T cell epitopes.

The cloning and expression of a γ-gliadin gene (pW1621) containing a defined epitope allowed the potential of a recombinant expression system to be evaluated. Protein extracted with aqueous ethanol and precipitated by the addition of salt from bacteria expressing this γ-gliadin gene migrated as a single band of the expected size. Moreover, the γ-gliadin was recognised by a T cell transfectant specific for the defined epitope,[9 15] indicating that this approach of expressing recombinant gliadins could be useful for the mapping of gliadin epitopes.

Several new α-gliadin sequences that were not present in the Swiss-Prot database were obtained in our study. This may indicate that there exist many more α-gliadin variants than those currently deposited in this database. The high number of distinct sequences may also reflect errors created during the PCR amplification of the genes. Two types of PCR errors can be envisaged. One is the misincorporation of nucleotides. However, based on the error rate found in a similar PCR with a γ-gliadin gene, this is likely to account for only a small part of the variability. A second error during the PCR could arise as partial products generated in one amplification cycle serve as primers in a subsequent cycle for distinct templates, thereby creating genes with shuffled sequences. To what extent this actually has taken place is difficult to ascertain. However, this should not be a serious flaw to the objective of using recombinant gliadins for characterisation of T

cell epitopes, as the latter consist of short peptide fragments of 10–15 amino acids for which the coding sequences are likely to be maintained.

The sequencing of 400 bases at the 5' end of 15 PCR clones amplified using the γ-gliadin specific primers identified seven sequences that were unrelated to gliadin while the remaining eight clones contained sequences encoding γ-gliadins. Full length sequencing of two of these clones showed one pseudogene and one full length gliadin which was successfully expressed.

The approach of expressing recombinant gliadins to characterise T cell epitopes has a limitation if a restricted number of distinct genes are represented. It is notable that the consensus sequence obtained from the α-gliadin clones was remarkably similar to that of A-gliadin,[22] with a discrepancy only in six positions. The A-gliadin sequence is essentially a biochemical "consensus" sequence obtained by amino acid sequencing of gliadin peptides. This suggests that the α-gliadin genes that we have described are broadly representative of the gliadin expressed naturally. This notion is also supported by a sequence cluster analysis of the deduced amino acid sequence of the genes cloned by us and the α-gliadin genes present in the Swiss-Prot database. The sequences identified by us were evenly distributed throughout the dendrogram (data not shown), indicating that the conditions we used for amplification and cloning of our α-gliadin genes did not result in the preferential cloning of a limited subset of gliadin genes.

We started by expressing nine different α-gliadin clones in two separate batches to obtain a broad representation of distinct sequences. As both batches were recognised in proliferation assays by four different T cell clones, we individually expressed the clones from one of them. Only seven out of nine α-gliadin clones yielded mature α-gliadin protein, and the amount of α-gliadin produced differed considerably among the different clones. This is most probably due to variation in the expression system, especially in the purification process of the recombinant gliadins. By testing each of the individual α-gliadins for recognition by T cell clones, we found that only one of the α-gliadin subtypes was recognised and that this was recognised by all four T cell clones tested. This indicates that most of the α-gliadin subtypes do not contain epitopes recognised by these T cells.

Our study shows that gliadins can be easily produced by recombinant DNA technology. Multiple proteins need to be expressed for T cell epitope characterisation, as T cells discriminate between distinct gliadins. The extra workload of expressing numerous gliadins is compensated for by a purification step, which is simple and efficient compared with the commonly used protocols for recombinant proteins that employ tags and affinity purification. This panel of recombinant gliadins, together with mass spectrometry analysis, should allow the efficient identification of T cell epitopes in gliadins.

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